Neuron
Article

Rapid Restoration of Cognition in Alzheimer's Transgenic Mice with 8-Hydroxy Quinoline Analogs Is Associated with Decreased Interstitial Aβ

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DOI 10.1016/j.neuron.2008.06.018

SUMMARY

As a disease-modifying approach for Alzheimer's disease (AD), clioquinol (CQ) targets β-amyloid (Aß) reactions with synaptic Zn and Cu yet promotes metal uptake. Here we characterize the second-generation 8-hydroxy quinoline analog PBT2, which also targets metal-induced aggregation of Aß, but is more effective as a Zn/Cu ionophore and has greater blood-brain barrier permeability. Given orally to two types of amyloid-bearing transgenic mouse models of AD, PBT2 outperformed CQ by markedly decreasing soluble interstitial brain Aß within hours and improving cognitive performance to exceed that of normal littermate controls within days. Nontransgenic mice were unaffected by PBT2. The current data demonstrate that ionophore activity, inhibition of in vitro metal-mediated Aß reactions, and blood-brain barrier permeability are indices that predict a potential disease-modifying drug for AD. The speed of recovery of the animals underscores the acutely reversible nature of the cognitive deficits associated with transgenic models of AD.

INTRODUCTION

Dysregulation of brain metal ion homeostasis, particularly Cu and Zn, may be closely involved in the pathogenesis of AD and its characteristic β-amyloid (Aß) neuropathology (Adlard and Bush, 2006). Zn ions are released presynaptically (Frederickson et al., 2006) and Cu ions postsynaptically (Schlief et al., 2005) into the glutamatergic synaptic cleft, where they could precipitate Aß and induce the formation of soluble toxic Aß oligomers (Barnham et al., 2004; Bush et al., 1994; Huang et al., 1999; Lee et al., 2002). Zn and Cu are indeed markedly enriched in plaque amyloid where they directly coordinate Aß (Dong et al., 2003; Opazo et al., 2002). While Cu and Zn pool in the extracellular plaque, the intracellular Cu stores are deficient in AD and could promote the generation of Aß (Bayer et al., 2003; Phinney et al., 2003), leading to a vicious cycle. Therefore, traditional high-affinity chelators that lower tissue metal levels are unlikely to be helpful in remedying amyloid pathology, and may actually worsen the biochemical features of Cu deficiency that characterize AD (Adlard and Bush, 2006).

Clioquinol (CQ) is an 8-OH quinoline with moderate affinity for Cu and Zn that inhibits metal-induced Aß aggregation and reactive oxygen species generation in vitro (Cherny et al., 2001). It lowers Aß production in cell culture through a mechanism that involves raising cellular metal levels, thereby increasing the levels of Aß-degrading matrix metalloproteinases (White et al., 2006). Oral administration of CQ (30 mg/kg/d) to Tg2576 mice, a transgenic (Tg) model for AD (Hsiao et al., 1996), reduced brain Aß burden by ~50% after 9 weeks, accompanied by a modest increase in brain Zn and Cu levels (Cherny et al., 2001). The effect of CQ treatment upon memory performance in Tg mice has not previously been reported. A pilot phase IIa clinical trial of CQ in AD patients showed evidence of slowing cognitive deterioration and significantly lowered plasma Aß42 levels (Ritchie et al., 2003). Further phase II/III studies were stalled by difficulties in preventing di-iodo 8-hydroxy quinoline contamination upon largescale chemical synthesis. Additional studies in Tg2576 mice and human volunteers showed that CQ entry into

the brain is limited, although upon brain entry it binds to amyloid plaques (Opazo et al., 2006).

PBT2 is also an 8-hydroxy quinoline, but lacks iodine; it was designed for easier chemical synthesis, higher solubility, and increased blood-brain barrier permeability (K. Barnham, E. Gautier, G. Kok, and G. Krippner 2003, 8-Hydroxy Quinoline Derivatives [World, Prana Biotechnology Ltd], PCT No. PCT/AU03/00914, Publication No. WO2004007461, patent pending). PBT2 was recently found to significantly reverse frontal lobe functional deficits and to significantly lower cerebrospinal fluid Aß42 in a 12-week phase IIa clinical trial in AD patients (Lannfelt et al., 2008).

Here we interrogate the screening procedure that we used to select PBT2 for clinical testing, since the mechanism of action of this drug could yield insights into AD pathogenesis. We show that PBT2 matched or exceeded the performance of CQ in a series of in vitro screening assays that we considered relevant to the potential therapeutic mode of action. We then compare the effects of PBT2 with those of CQ on behavior and neuropathology in two well-established amyloid protein precursor (APP) Tg mouse models of AD, the APPswe/PS1 dE9 (APP/PS1) (Jankowsky et al., 2001) and Tg2576 (Hsiao et al., 1996) models. Both types of Tg mice overexpress APP bearing missense mutations that cause AD in humans, but the APP/PS1 model additionally expresses human presenilin with a deletion mutation (dE9) that also causes AD. Both lines have progressive spatial learning deficits that are accompanied by rising cerebral Aß levels and increasing amyloid plaques commencing at about 12 months of age for Tg2576 and 6 months of age for the APP/PS1 model. Apart from testing cognitive outcomes, experiments in two models allow us to determine whether specific forms of Aß change in register with cognitive improvement in both strains. This is important because cognitive loss in APP Tg mice and in AD itself is not a simple product of increased Aß concentration or precipitate formation (McLean et al., 1999).

RESULTS

PBT2 Is a Superior Ionophore to CQ

Since recent findings suggest that increasing intracellular Cu or Zn may lower Aß production (Bayer et al., 2003; Borchardt et al., 1999; Cater et al., 2008; Phinney et al., 2003; White et al., 2006), we appraised PBT2 and CQ for their ionophoric ability to promote the transport of Cu, Zn, and Fe across cell membranes. PBT2 promoted markedly more uptake of Zn and Cu than CQ in cell culture (Figure 1).

PBT2 Inhibits Aß:Cu-Mediated Redox Chemistry and Synaptotoxicity In Vitro

We next assessed the ability of the compounds to mitigate the effects of excess metal binding to Aß (hypermetallation) in vitro. Both CQ and PBT2 significantly reduced the production of hydrogen peroxide by Aß:Cu complexes (Puglielli et al., 2005) (CQ, −49.9%, $p < 0.0001$; PBT2, −59.2%, $p < 0.0001$), with PBT2 providing a significantly greater inhibition than CQ ($p = 0.01$) (Figure 2A). Both compounds were effective in the dissolution of Zn-induced Aß1-42 precipitates (Huang et al., 1997) (Figure 2B), with CQ significantly more effective than PBT2 at 10 μM. Both compounds markedly inhibited Cu-induced Aß dityrosine crosslinking (Figure 2C). PBT2 treatment of APP/PS1 brain slices resulted in a significant reduction in Zn detected in thioflavin-positive plaques, as compared with buffer-treated slices or slices treated with a quinoline that does not coordinate metals, 3-aminoquinoline (3-AQ) (Figure 2D). PBT2 and CQ both significantly reversed the Aß-induced inhibition of long-term potentiation (LTP) in hippocampal slices (Figure 3).

PBT2 Reverses the Alzheimer Phenotype within Days

PBT2 was well tolerated at doses up to 30 mg/kg/d, and for an equivalent oral dose in mice (30 mg/kg), the brain-to-plasma ratio of PBT2 was 24- to 31-fold higher than that of CQ, which may be attributed to the latter's lower plasma protein binding (Table 1). Initially we tested PBT2 at 30 mg/kg/d by gavage for 9 weeks, as this was the protocol originally used for CQ (Cherny et al., 2001). We found that PBT2 significantly decreased insoluble Aß levels by ~30% ($p = 0.04$), soluble Aß by 37% ($p = 0.006$), and plaque burden by ≈80% ($p = 0.0008$) in 15-month-old Tg2576 mice (See Supplementary experimental procedures and Figure S1, available online). By comparison, CQ decreased insoluble Aß levels by 49%, increased soluble Aß levels by 52%, and decreased plaque burden by 25% in 21-month-old Tg2576 mice (Cherny et al., 2001). PBT2 treatment did not alter metal levels (Cu, Zn, Mn, or Fe) in the brain, liver, kidney, or plasma, establishing that, like CQ, PBT2 is not acting as a broad-based chelator. Having established that PBT2 had comparable efficacy to CQ in a 9 week protocol, we next studied how rapidly the onset of Aß modulation occurred.

We tested the effects of CQ and PBT2 upon extracellular brain Aß levels using in vivo microdialysis (IVM). Female APP/PS1 mice (22 months of age) were given a single oral dose of either PBT2 (30 mg/kg, n = 3) or CQ (30 mg/kg, n = 2) following baseline collections of interstitial fluid. Soluble interstitial Aß ($Aß_{ISF}$) decreased by ≈30%–40% over the 20 hr following treatment with a single oral dose of CQ (Figure 4A). PBT2-treated animals showed a markedly greater (≈80%) decrease in $Aß_{ISF}$ (Overall ANOVA, $p = 0.05$), with significant decreases at 8 (−50%, $p = 0.03$), 16 (−55%, $p = 0.03$), and 20 (−81%, $p = 0.0001$) hr posttreatment, as compared with baseline values (Figure 4A). To confirm the effect of PBT2 on $Aß_{ISF}$ levels, we repeated the IVM experiment in male Tg2576 mice (~18 months old, n = 5). A single oral dose of PBT2 (30 mg/kg) resulted in a significant decrease in $Aß_{ISF}$ at 8 (−34%, $p = 0.0003$), 12 (−33%, $p = 0.0001$), and 16 (−32%, $p = 0.01$) hr posttreatment, as compared with baseline values (Figure S2A). To gauge the entry of PBT2 into the brain interstitium, we measured the levels in microdialysate in a parallel study of normal male, 12-week-old, C57Bl/6 mice (n = 5). Levels of PBT2 peaked at 4 hr postgavage, and were not detectable by 12 hr (Figure S2B). The decrease in $Aß_{ISF}$ in the Tg mice treated with PBT2 therefore began following an ≈2–4 hr lag to compound exposure. Although these experiments were compared with baseline readings from within the same animals, we also determined that $Aß_{ISF}$ levels are normally stable in untreated female APP/PS1 (n = 5) and untreated male Tg2576 (n = 9) mice over the same intervals (Figure S2C).

These data demonstrate that PBT2 and CQ decrease soluble $Aß_{ISF}$ levels within hours. To gain further insight into possible

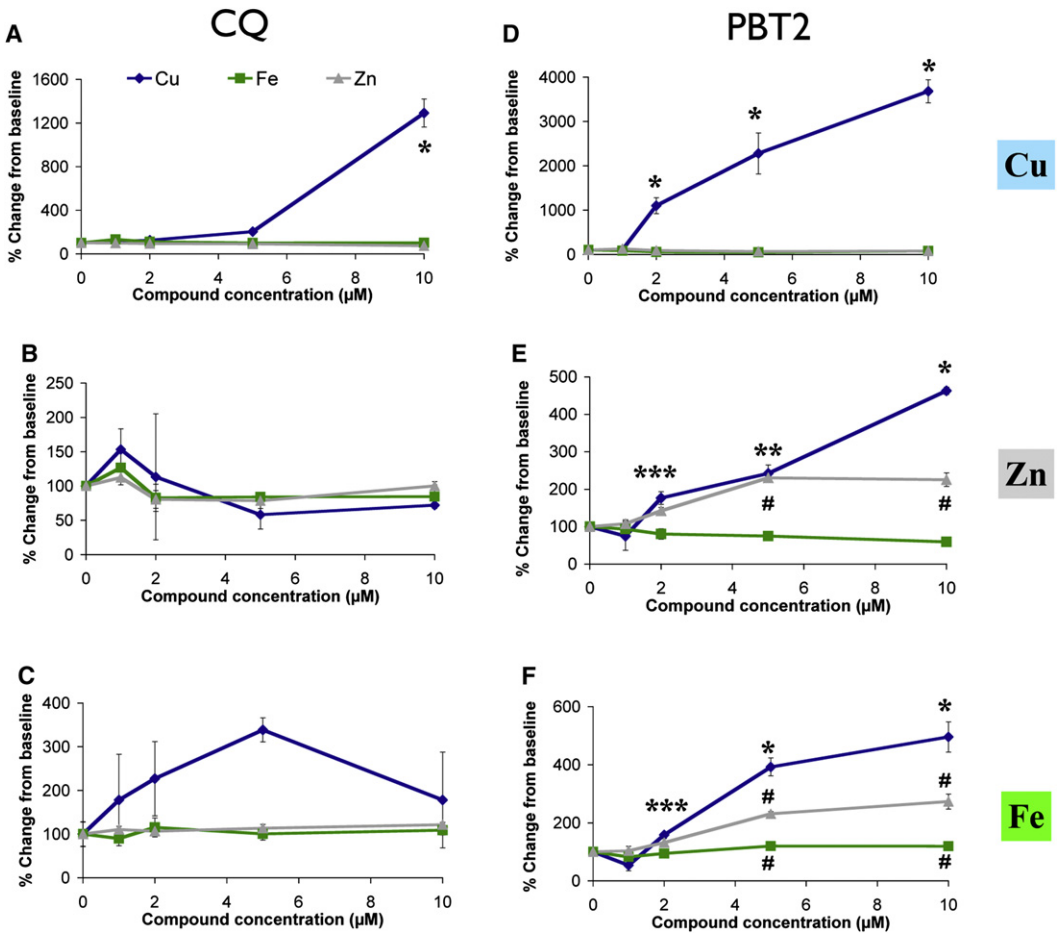

Figure 1. Ionophore Activity of CQ and PBT2

M17 human neuroblastoma cells were treated with 10 μM of Cu (A and D), Zn (B and E), or Fe (C and F) in the presence of varying concentrations of CQ (A–C) or PBT2 (D–F), and cellular metal levels were measured after 5 hr. Data are means ± SEM; n = 3. *p < 0.05 for all comparisons; #p < 0.05 for comparisons to 0, 1, and 2 μM; **p < 0.05 for comparisons to 0 and 1 μM; ***p < 0.05 for comparisons to 1 μM.

mechanisms for the PBT2 effect, we repeated the IVM experiments in young APP/PS1 mice (n = 4, 3–4 months of age). These animals, which lack the overt extracellular Aß deposits seen in the older animals used in this study, also showed a significant reduction in $Aß_{ISF}$ levels over the 20 hr following a dose of PBT2 (30 mg/kg) (Figure 4B). The drop in $Aß_{ISF}$ commenced 4 hr earlier than in the older animals. We hypothesize that in young animals without amyloid deposits, the clearance of Aß is more prompt upon treatment with PBT2, since there is no dissociable reservoir of precipitated Aß to replenish $Aß_{ISF}$ (Figure 7).

Evidence indicates that soluble extracellular Aß can affect cognition (Cleary et al., 2005; Townsend et al., 2006). Since our IVM data showed that PBT2 induces a rapid drop in $Aß_{ISF}$, we investigated the effects of PBT2 and CQ treatment over a short time frame in the Tg mice by testing them on the Morris Water Maze (MWM). We treated 8-month-old female APP/PS1 mice with CQ (30 mg/kg/d, n = 7), PBT2 (30 mg/kg/d, n = 7) or vehicle (Standard Suspension Vehicle [SSV], n = 18) for a total period of 11 days (Table S1A) and monitored effects on the MWM. The APP/PS1 mice exhibited marked and significant behavioral improvement compared with SSV-treated Tg mice within 6 days of treatment with PBT2, and showed both significantly enhanced acquisition of the spatial learning task (Overall ANOVA $F_{1,23}$ = 35.107, p < 0.0001, Figure 5A; for path length analysis see Figure S3A; for comparison of tracking profiles between Day 1 and Day 6 see Figure S4; also see Movies S1 and S2) and enhanced retention of the task in a 24 hr probe trial (p = 0.008, Figure 6B; for full quadrant analysis see Figure S3B). There was also a significant improvement for CQ-treated animals in the acquisition task (Overall ANOVA $F_{1,23}$ = 16.195, p = 0.0005), but the retention scores were not significantly different from those of controls (p = 0.2, Figures 5A and 5B). The PBT2-treated animals, compared with CQ-treated animals, performed significantly better on the acquisition (Overall ANOVA $F_{1,12}$ = 6.131, p = 0.03) and retention (p = 0.05) of the task. We also treated non-Tg littermates with CQ (30 mg/kg/d, n = 6), PBT2 (30 mg/kg/d, n = 6), or vehicle (SSV, n = 7). Neither CQ nor PBT2 had an effect on the performance of wild-type (WT) animals in the acquisition or retention (Figures 5C and 5D) of the MWM, demonstrating that the improvement in Tg mice was specific to the Alzheimer

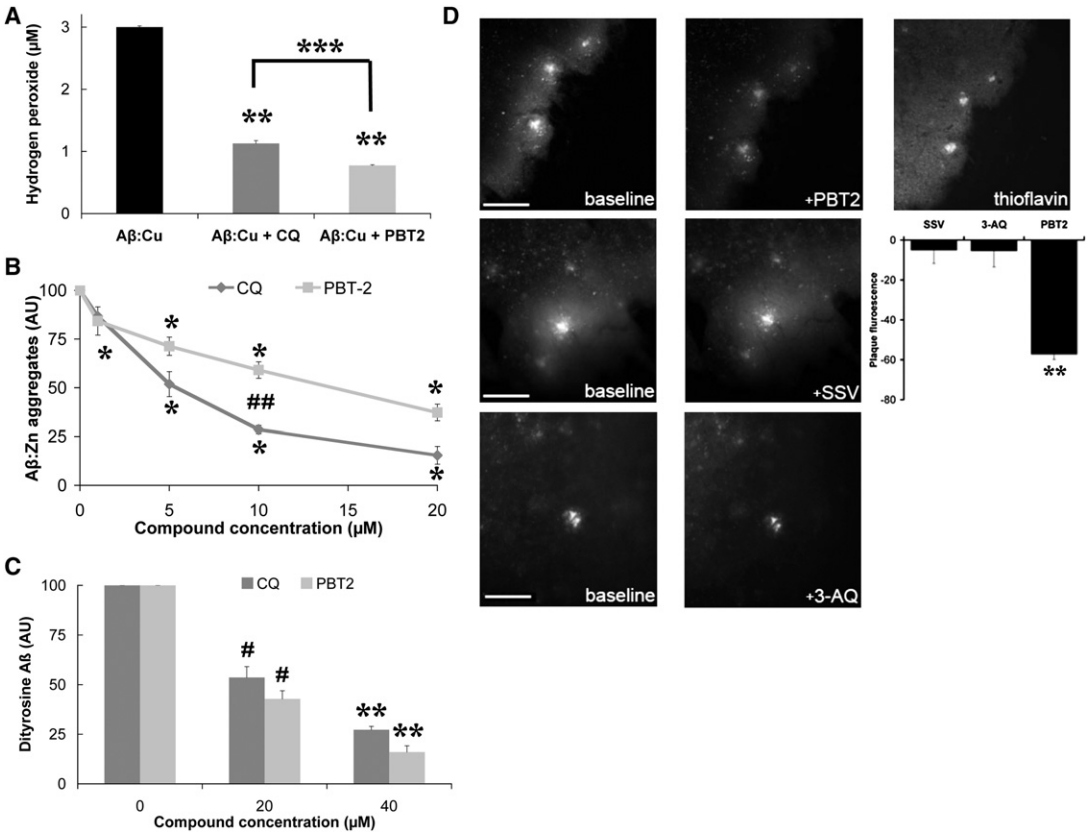

Figure 2. CQ and PBT2 Inhibit the Effects of Aß Hypermetallation In Vitro

(A) Inhibition of H_2O_2 generation. Effects of CQ and PBT2 (both 0.5 μM) upon H_2O_2 generation by Aß1-42:Cu (100 nM:200 nM) complexes in the presence of dopamine (5 μM) are shown. (B) Dissolution of Zn-induced Aß1-42 precipitates. After incubation with $ZnCl_2$ to induce aggregation, Aß1-42 aggregates were incubated for 2 hr with CQ or PBT2, and residual aggregation was assayed by ThT fluorescence. Data are fluorescence levels normalized (100 AU) to the absence of compound. (C) CQ and PBT2 inhibit Cu-mediated dityrosine crosslinking of Aß1-40 (9 kDa dimer) assayed by densitometric quantification of western blots probed with anti-dityrosine antibody. Data are densitometry levels normalized (100 AU) to the absence of compound. For (A–C), *$p < 0.02$ to baseline; **$p < 0.001$ to baseline; #$p < 0.05$ to baseline; ##$p < 0.05$ between concentrations; ***$p < 0.02$. All data are means ± SEM; n = 3. (D) Reduction of plaque-bound Zn by PBT2 treatment. Plaques in brain slices (200 μm) from an APP/PS1 mouse were stained with Zinpyr-4 and then treated with PBT2 (n = 17), SSV control solution (n = 7), or the nonchelating quinoline 3-aminoquinoline (3-AQ, n = 12) for 5 min. Individual plaques were measured for signal intensity of this fluorophore before and after drug treatment. There was a significant loss of Zn signal within thioflavin-positive plaques upon PBT2 treatment as compared with controls (**$p < 0.001$ PBT2 compared with either control).

phenotype. Surprisingly, the PBT2-treated APP/PS1 mice were superior to untreated non-Tg littermates in the acquisition of the task ($F1,12 = 12.25$, $p = 0.004$) (Figure 5A). There were no significant differences on the retention of the task. Comparison of sham-treated WT and sham-treated Tg animals revealed that, as expected, the Tg mice had deficits on the acquisition (ANOVA $F1,23 = 5.74$, $p = 0.02$, Figure 5A) and retention ($p = 0.05$) of the task, as compared with aged-matched control animals.

The most robust behavioral improvements were seen in animals treated with PBT2. To explore the limiting dose required to achieve this cognitive benefit and to determine whether improvements were possible in both genders, we utilized the same experimental paradigm as above and treated male APP/PS1 mice with either PBT2 at a lower dose (10 mg/kg/d, n = 6) or vehicle (SSV, n = 7) (Figures 5E and 5F). An overall improvement in the acquisition of the task was observed after the short-term 11 day trial ($F1,10 = 6.64$, $p = 0.028$, Figure 5E), 24 hr after which there were no differences in the retention scores between the two groups (data not shown). In order to determine if these benefits were sustained, animals were maintained on PBT2 for a total of 35 days, during the last week of which they were reassessed on the MWM (see protocol in Table S1B). PBT2-treated animals continued to show a significant improvement in the acquisition of the task across the whole study (Overall ANOVA $F1,11 = 7.12$, $p = 0.022$, Figure 5E), and the probe trial conducted 24 hr after the last 6 days of trials (Figure 5F) revealed a trend toward improved retention of the task in the low-dose PBT2-treated animals, compared with SSV-treated controls. Data such as these, where a significant improvement is found in the acquisition of the task, but not the retention of the task, may either represent a specific effect on learning, but not recall, or it may be a consequence of the cohort being too small to reveal the effect on recall. The trend observed suggests that a larger cohort may have revealed significant effects of PBT2 treatment on recall at 10 mg/kg.

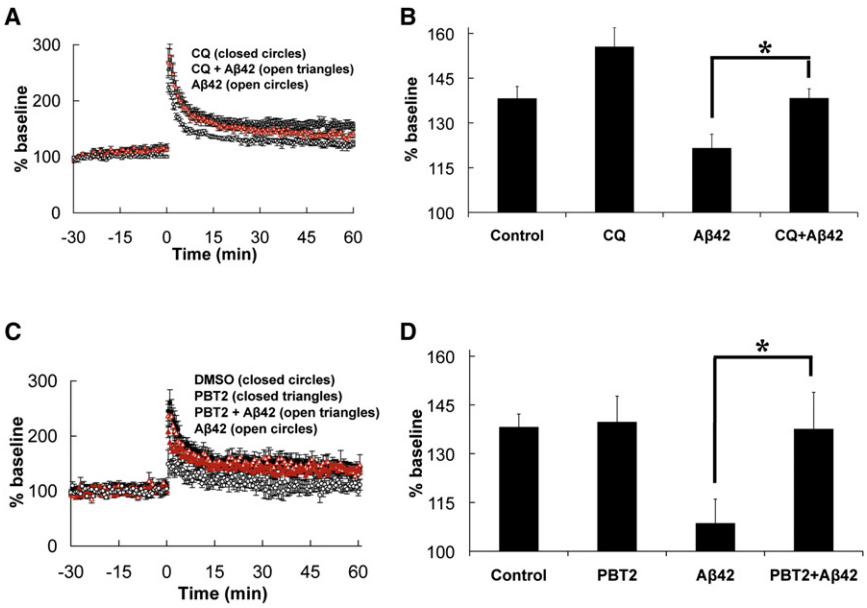

Figure 3. CQ and PBT2 Prevent Aß-Induced Inhibition of LTP in Hippocampal Slices

(A and C) Field potential recordings of mouse hippocampal slices, CA1 region, showing the effects of stimulation upon field excitatory postsynaptic potentials (fEPSPs) expressed as mean percentage of baseline (n = 12) ± SEM. The effects of 2 μM Aβ42 ± 2 μM PBT2 or 4 μM CQ on fEPSPs following tetanic stimulation is shown. PBT2 and CQ were studied at the highest concentrations that had no effect on LTP. The presence of DMSO at the concentrations used in aCSF had no effect on fEPSPs or LTP. (B and D) Percent change in fEPSPs (±SEM) relative to baseline averaged from data taken 55 to 60 min posttetanus. *p < 0.05, unpaired t test.

We also tested the benefit of PBT2 on learning and memory in a second type of APP Tg model. MWM studies using Tg2576 mice (female) demonstrated similar results to those in APP/PS1 mice, with the PBT2-treated animals (n = 9) showing a significant improvement in performance as compared with SSV-treated animals (n = 9) across the task (Overall ANOVA F1,15 = 4.9, p = 0.04) (Figure S5A), although the magnitude of the effect (53% difference on trial 6) was not as large as that seen in the APP/PS1 animals (85% difference on trial 6). While the PBT2-treated Tg2576 animals demonstrated ~100% improvement in the probe trial as compared with SSV-treated controls (Figure S5B), this did not reach statistical significance (p = 0.18).

We assessed the brain tissue of a subset of the mice (n = 7 PBT2-, n = 7 CQ-, and n = 13 SSV-treated APP/PS1 mice) studied in the MWM experiments to determine whether the cognitive improvements were associated with alterations in AD-related parameters such as Aß burden, phosphorylation of tau, and synaptic protein loss. We assayed insoluble Aß burden in the APP/PS1 animals using surface-enhanced laser-desorption ionization time of flight (SELDI-TOF) mass spectrometry, which allows for the quantitation of individual Aß fragments. These data (Figure 6) demonstrate that insoluble Aß40 (30% reduction, p = 0.06), Aß42 (41% reduction, p = 0.03), and Aß43 (35% reduction, p = 0.11) were reduced with 11 days of PBT2 treatment (30 mg/kg/d), whereas there was no concomitant effect on insoluble Aß in the CQ-treated animals. We also assessed soluble oligomeric Aß by western blot, utilizing oligomer-specific antibody A11. This demonstrated a significant reduction in oligomeric Aß in PBT2-treated animals (p < 0.05) and a significant increase in CQ-treated animals (p < 0.05) as compared with SSV-treated controls (Figure S6). Neither CQ nor PBT2 treatment affected APP levels (data not shown).

We also assessed the effect of PBT2 treatment in the Tg2576 mice that were studied with the MWM. In contrast to the APP/PS1 mice, different species of insoluble Aβ assayed by SELDI-TOF mass spectrometry decreased as a result of drug treatment (Figure S7A). Significant decreases with drug treatment (30 mg/kg/d, 11 days treatment) were observed in Aß1-37 (73%, p = 0.03), Aß1-39 (79%, p = 0.04) and Aß2-46 (92%, p = 0.02). Reductions in Aß1-40 (48%, p = 0.1) and Aß1-42 (25%, p = 0.49), as well as oxidized Aß1-42 (64%, p = 0.3), were also seen with drug treatment, but did not reach statistical significance (due to an outlier). In contrast to APP/PS1 animals, there was no significant change in soluble oligomeric Aß detected by A11 western blot (Figure S7B). Taken together, these data indicate that $Aß_{ISF}$ is the only brain Aß species we could detect that changes in register with cognitive improvement in both Tg strains upon PBT2 treatment.

Table 1. Brain and Plasma Concentrations of CQ and PBT2 (30 mg/kg) in WT and Tg Mice at 120 Min after Oral Administration

Strain	Compound	Brain Concentration (ng/g)	Plasma Concentration (ng/ml)[a]	Brain:Plasma Ratio
WT (female Bl6/SJL)	CQ	161.0 ± 56.4	1084.1 ± 194.4	0.15 ± 0.06
	PBT2	796.0 ± 513.5	216.9 ± 97.7	3.63 ± 1.31
Tg2576 (female Bl6/SJL)	CQ	122.6 ± 25.2	1143.2 ± 343.8	0.12 ± 0.06
	PBT2	318.1 ± 75.1	85.0 ± 14.9	3.74 ± 0.68

All data are presented as mean ± SD (n = 4 or 5). PBT2 exhibits much greater uptake into the brain, as compared with CQ, in both WT and Tg2576 animals.

[a] Plasma protein binding is >99.6% (n = 2) for CQ and 95.1% ± 0.8% (mean ± SD, n = 3) for PBT2.

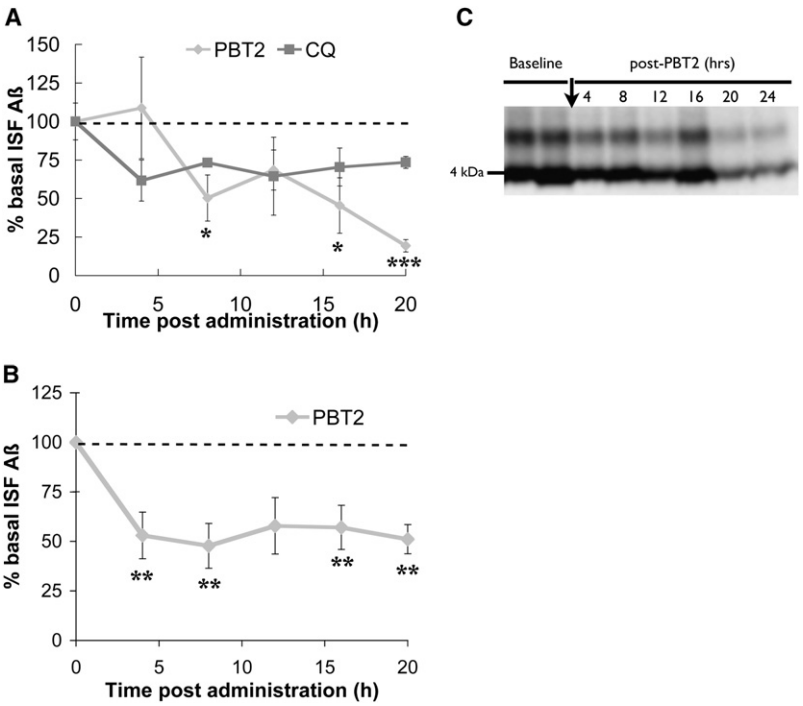

Figure 4. PBT2 Reduces Interstitial $Aß_{ISF}$ Levels in APP/PS1 Mice, as Ascertained by In Vivo Microdialysis

(A) $Aß_{ISF}$ was assayed by western blot densitometry in dialysates collected (4 hr collection bins) from the hippocampi of 14- to 24-month-old male and female APP/PS1 mice (CQ, n = 3; PBT2, n = 3). Animals were administered a single gavage dose of either CQ (30 mg/kg) or PBT2 (30 mg/kg), and dialysates were collected over the subsequent 20 hr. The data are normalized to the average $Aß_{ISF}$ concentration for the individual animal measured over the 8 hr prior to administration of the ionophore (mean Aß concentration for the group = 1.99 μg/l).

(B) $Aß_{ISF}$ was assayed by western blot densitometry in dialysates collected (4 hr collection bins) from the hippocampi of 3- to 4-month-old female APP/PS1 mice (n = 4). The data are normalized as in (A) (mean Aß concentration for the group = 0.47 μg/l). Animals were gavaged with PBT2 (30 mg/kg) and dialysates were collected over the subsequent 20 hr.

(C) A representative western blot for Aß (arrow indicates time of PBT2 administration).

Data are means ± SEM; one-way ANOVA, *p < 0.05, **p < 0.01 compared with baseline, ***p < 0.0001 compared with baseline.

PBT2 Affects Both Tau Phosphorylation and Synaptophysin Protein Levels

Hyperphosphorylation of tau and its accumulation in neurofibrillary tangles are also hallmark biochemical features of AD (Grundke-Iqbal et al., 1986; Ihara et al., 1986). We assessed both total tau and tau phosphorylated at Ser396, an epitope that is abnormally phosphorylated in AD and which promotes tau polymerization (Abraha et al., 2000), by western blot in the APP/PS1 mice treated for 11 days in this study to gauge the association of changes in these proteins with the observed improvements in cognition. Our data demonstrated a significant reduction in phosphorylated tau in the animals treated for 11 days with PBT2 (soluble fraction: −56%, p = 0.005; insoluble fraction: −38%, p = 0.01), which was not recapitulated in the CQ-treated animals (insoluble: −8%, p = 0.6; soluble: +81% increase, p = 0.02) (Figures 6C and 6D). Neither treatment elicited a change in total tau levels (not shown). Longer-term treatment (35 days) with a lower dose of PBT2 (10 mg/kg/d) in the APP/PS1 mice resulted in a sustained decrease in soluble phosphorylated tau (−26%, p = 0.03), but the decrease in insoluble phosphorylated tau was not significant (−27%, p = 0.4) (Figures 6E and 6F). There were again no significant changes in levels of total tau (not shown).

We also examined tau levels in the Tg2576 mice that were treated with PBT2 (30 mg/kg/d) for 11 days. Unlike the effects observed in the APP/PS1 model, significant changes in total tau were observed. Total insoluble tau levels were decreased with PBT2 treatment (−24%, p = 0.003) while soluble tau was increased (31%, p = 0.0006) (Figure S7C). In contrast, phosphorylated tau levels were not significantly different between treatment groups (Figure S7D). Therefore, there was no concordance between changes in tau and Ser396 phosphorylated tau levels in the two treated Tg mouse models and the observed improvements in cognition.

Synaptophysin is a presynaptic marker that shows a regional decrease of up to 50% in AD (Callahan et al., 1999; Sze et al., 1997), which may be related to the accumulation of synaptotoxic Aß species. We examined brain synaptophysin levels by western blot in APP/PS1 animals given 30 mg/kg/d of either PBT2 or CQ for 11 days. Both treatments significantly augmented the levels of synaptophysin, with PBT2 (63%, p = 0.001) inducing a greater increase than CQ (44%, p = 0.01) (Figure 6H). The lower dose of PBT2 (10 mg/kg) in the APP/PS1 mice did not elicit a significant change in synaptophysin levels (3%, p = 0.8, data not shown). Synaptophysin levels were also significantly increased (38%, p = 0.03) in the Tg2576 mice treated with 30 mg/kg PBT2 for 11 days as compared with the SSV-treated controls (Figure S7E).

DISCUSSION

We found that oral treatment with compounds based on the 8-OH quinoline scaffold induced a dramatic improvement in learning and memory in Tg models of AD, accompanied by marked inhibition of AD-like neuropathology. These outcomes were rapid, with $Aß_{ISF}$ reduction occurring within hours, and significant cognitive benefits were seen within days of the first administration of compound.

While it has been previously shown that the responsible brain lesions in these AD models are indeed reversible (Kotilinek et al., 2002), the speed of the cognitive recovery upon treatment in our studies is consistent with a lesion that could be analogous to acute intoxication causing delirium. AD is not considered an acute brain syndrome, but the possibility that there is a component of delirium in the otherwise chronic disorder could explain

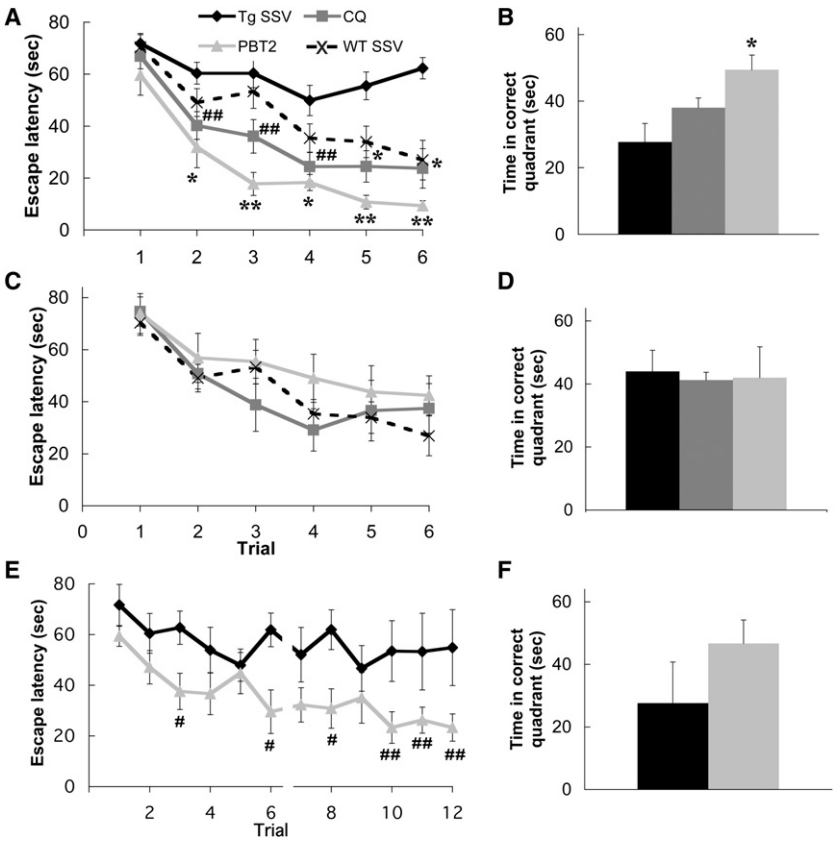

Figure 5. PBT2 Improves the Performance of APP/PS1 Transgenic Mice, but Not Nontransgenic Littermates, in the MWM

(A) Eight-month-old female APP/PS1 mice were treated with SSV (n = 18), CQ (30 mg/kg, n = 7), or PBT2 (30 mg/kg, n = 7) for a total period of 11 days (see Table S1A), during which their performance on the MWM was assessed. PBT2-treated animals showed a significant improvement in the acquisition of the task (ANOVA, $p < 0.0001$), as did CQ-treated animals (ANOVA, $p = 0.0005$). PBT2-treated animals performed significantly better than CQ-treated animals ($p = 0.03$) and also significantly better ($p = 0.004$) than SSV-treated age-matched WT controls (dashed line). SSV-treated Tg animals demonstrated significant deficits ($p = 0.02$) on this task as compared with SSV-treated WT controls.

(B) Twenty-four-hour retention task. Retention was assessed by the amount of time spent in the quadrant where the escape platform was present for the acquisition task. A significant improvement is shown in PBT2-treated animals only ($p = 0.008$).

(C and D) Eight-month-old female WT littermates were also assessed, and neither CQ (n = 6) nor PBT2 (n = 6) had any effect on performance in the task, as compared with SSV (n = 7).

(E and F) Effects of low-dose PBT2 (10 mg/kg, n = 6) compared with SSV control (n = 7) in male APP/PS1 mice over a total period of 35 days (see Table S1B for trial regimen; the line breaks represent a break in trial tests). PBT2-treated animals showed an enhanced acquisition of the task (ANOVA, $p = 0.022$) and a nonsignificant improvement in the retention task. Data are mean times ± SEM; t test against same-trial SSV control, *$p < 0.003$, **$p < 0.0002$, #$p < 0.01$, ##$p < 0.05$.

some well-known features of the disease, such as the rapid fluctuations in cognitive performance (Ballard et al., 2001; Eikelenboom and Hoogendijk, 1999). In this regard, it is interesting that commonly used anesthetics such as isoflurane, which is associated with postoperative delirium, are a risk factor for AD, and induce Aβ production and apoptosis in vitro, which is rescued by CQ (Xie et al., 2007; Zhang et al., 2008). Our findings underscore the possibility that acute intoxication is a feature of Tg mouse models for AD, and may be an underappreciated facet of the human disorder. From our current findings, the toxic mediator of the acute cognitive deficits was most likely $Aß_{ISF}$ (Figures 4 and S2), which was conspicuously decreased by treatment in both strains of Tg mice and in approximate register with the short time frame of cognitive improvement. This is in agreement with previous studies that have implicated $Aß_{ISF}$ in disease pathogenesis (Cirrito et al., 2005; DeMattos et al., 2004). While we also observed decreases in other Aß species, including Aß1-40 and Aß1-42, the decreases with PBT2 treatment were not statistically significant across both Tg animal models. Thus, the data presented here must be interpreted with the caveat that we cannot eliminate the possibility that factors other than $Aß_{ISF}$ have contributed to the rapid restoration of cognition in our model. In addition to the changes in Aß, there were further biochemical changes that may also have contributed to this cognitive benefit. Notably, synaptophysin levels were also increased in both Tg lines over the period of treatment (Figure 6), supporting the likelihood that synaptotoxicity had been reversed. We also observed a decrease in phosphorylated tau levels (Figure 6), although this only occurred in the APP/PS1 mice and could not, therefore, explain the cognitive improvement in Tg2576 animals.

The current data allow us to deduce valuable information about factors that have increased the potency of PBT2 over its predecessor CQ, which help elucidate the mechanism of action. The in vitro screening protocols were based on a theory that a candidate drug needs to neutralize the consequences of the hypermetallation of Aß by Cu and Zn. To this end, both CQ and PBT2 block the catalytic generation of H_2O_2 by the Aß:Cu complex (Figure 2A), reverse Zn-mediated aggregation of synthetic Aß (Figure 2B), and inhibit the formation of redox-mediated covalently crosslinked soluble oligomers of Aß (Figure 2C). However, neither compound was clearly superior in these in vitro assays in a manner that could explain why PBT2 was more effective than CQ in its cognitive benefits in Tg mice (Figure 5) and in decreasing interstitial Aß (Figure 4) and insoluble Aß (Figure 6) in this short time frame. Increased penetration of the blood-brain barrier, possibly due to lower plasma protein binding, is certainly one feature that is likely to explain the increased potency of PBT2, although factors other than pharmacokinetics and blood-brain barrier penetration are also likely to have contributed. Another in vitro assay that clearly differentiated PBT2 as superior to CQ was the ionophore assay (Figure 1). We previously reported that CQ decreased Aß levels in neuronal cells by acting as

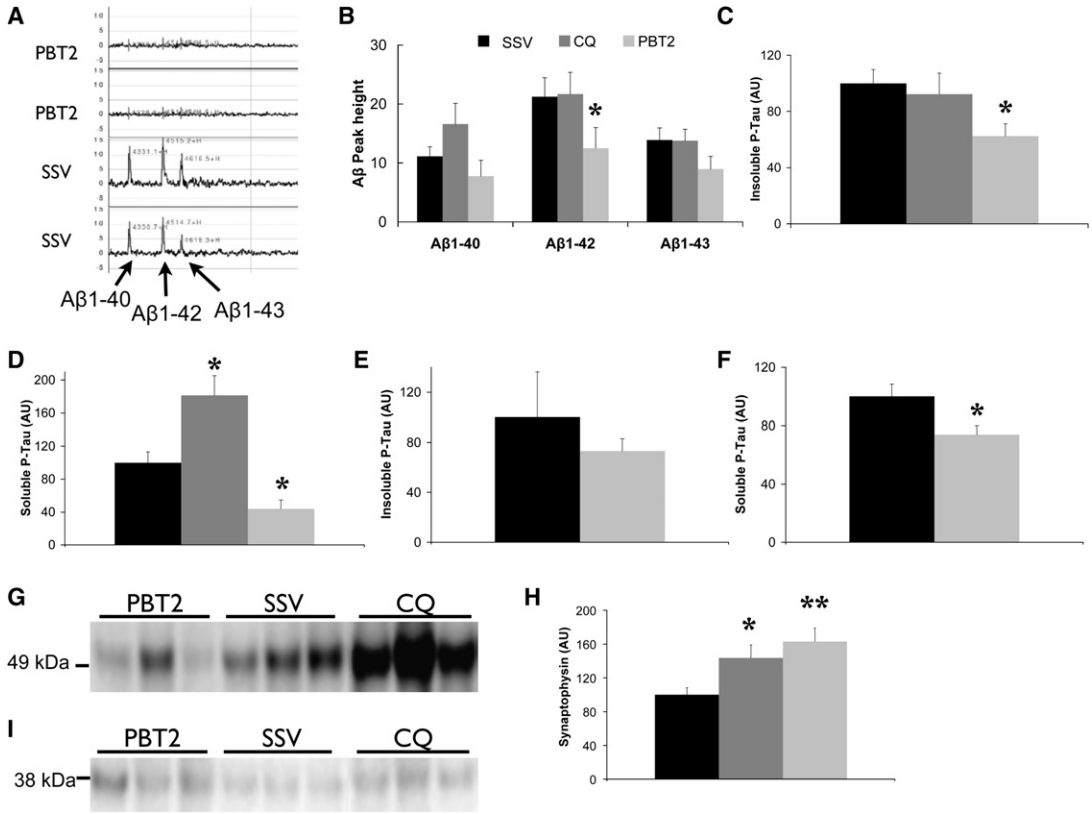

Figure 6. PBT2 Alters AD-Related Brain Biochemistry in APP/PS1 Mice

PBT2 decreases Aß burden in APP/PS1 mice by SELDI-TOF mass spectrometry. (A) Sample spectra. (B) Quantitated data are average peak heights (±SEM) in all animals. PBT2-treated animals demonstrate significantly lowered Aß1-42. *p = 0.03, t test, compared with SSV control. PBT2 decreases Ser396 phosphorylated tau levels in APP/PS1 mice. Ser396 phosphorylated tau content was assayed by western blot and quantified by densitometry (normalized to SSV levels, 100 AU). Levels of (C) insoluble phosphorylated tau and (D) soluble phosphorylated tau in APP/PS1 mice treated for 11 days with CQ or PBT2 (30 mg/kg/d) showing significant reductions with PBT2 treatment. Levels of (E) insoluble phosphorylated tau and (F) soluble phosphorylated tau in APP/PS1 mice treated for 35 days with PBT2 (10 mg/kg/d) showing significant reductions with PBT2 treatment. (G) Representative western blot of phosphorylated tau in the soluble brain fractions. *p < 0.05, t test, compared with SSV control. Data are means ± SEM. CQ and PBT2 increase synaptophysin levels in APP/PS1 mice. (H) Synaptophysin content was assayed by western blot and quantified by densitometry (normalized to SSV levels, 100 AU), and showed significant increases with both CQ and PBT2 treatment. (I) Representative western blot. Data are means ± SEM for all animals. *p = 0.03, **p = 0.009, t test, compared with SSV control.

a Cu/Zn ionophore (White et al., 2006). Therefore, we suspect that the superior ability of PBT2 to transport Cu or Zn ions into the cell reflects a critical property of this class of potential therapeutic. Our in vitro assays cover multiple potential mechanisms by which our candidate drugs could lower or detoxify brain Aß. However, based upon our current findings, we propose the following heuristic model for the mechanism of action of these compounds (Figure 7).

We hypothesize that PBT2 and related compounds capture metal ions from oligomerized and precipitated interstitial Aß, perhaps forming redox-neutral ternary complexes (Figure 7). The consequent dissolution of oligomerized Aß facilitates the clearance of amyloid. PBT2 or CQ could also bring metals captured from Aß oligomers into nearby cells, which could then increase matrix metalloproteinase expression and thereby foster clearance or degradation of $Aß_{ISF}$ (White et al., 2006) (see proposed mechanism of action, Figures 7 and 2D). Similarly, liberated Zn and Cu may restore the activities of interstitial metalloproteinases that degrade $Aß_{ISF}$ (Strozyk et al., 2007). We hypothesize that the clearance of extracellular Aß resolves neuronal intoxication from pro-oxidant products like H_2O_2 (Figure 7), and so leads to elevated synaptophysin (Figure 6) and improved synaptic function (Figure 5). Additionally, the normalization of Zn and Cu reuptake in the glutamatergic synapse may improve the function of the NMDA receptor and restore LTP.

The ionophoric activity of CQ has been shown to activate PI3K, ERK1/2, and JNK and also promote the phosphorylation of glycogen synthase kinase-3; therefore, treatment with this class of compound may decrease the hyperphosphorylation of tau (White et al., 2006) (Figures 6C, 6D, and 7). Recent findings have indicated that decreasing brain tau levels in APP Tg mice uncouples Aß from downstream pathogenic mechanisms and improves cognitive performance (Roberson et al., 2007). While Roberson et al. (2007) did not differentiate between solution states of brain tau, we found that PBT2 (but not CQ) decreased insoluble and soluble phosphorylated tau in the APP/PS1 mice (Figure 6) and insoluble total tau in the Tg2576 mice (although soluble total tau was also increased in the Tg2576 model

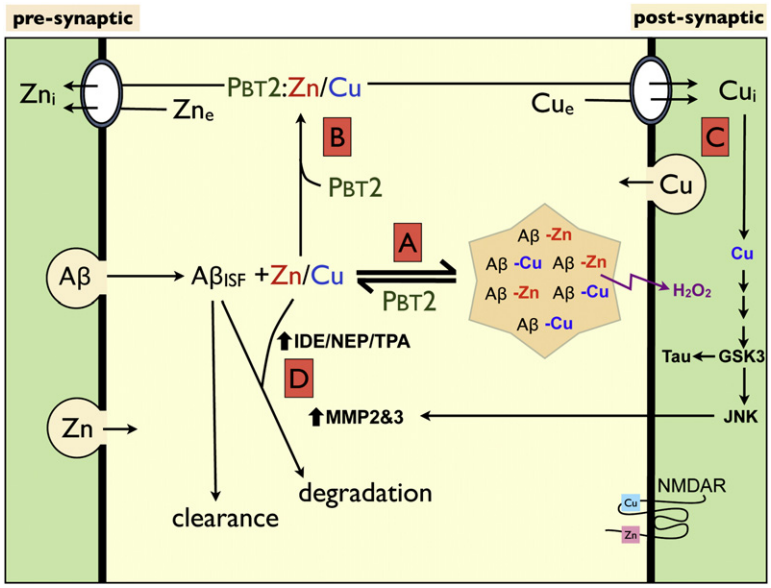

Figure 7. Proposed Mechanism of Action of PBT2

The figure shows a notional glutamatergic synaptic cleft with Aß, Zn, and Cu being constitutively released from presynaptic and postsynaptic origins. Zn and Cu are in an ionic form and normally modulate NMDA receptor function (Frederickson et al., 2006; Schlief et al., 2005). A. Soluble interstitial Aß ($Aß_{ISF}$) reacts with extracellular Zn and Cu to form protease-resistant Aß oligomers and aggregates, which are in dissociable equilibrium with the soluble species. PBT2 reacts with accessible Zn and Cu, driving the dissociable equilibrium to the left, and promoting soluble species. B. PBT2 also promotes the clearance of Zn and Cu from the cleft, possibly by accessing the established energy-dependent metal reuptake mechanisms that appear to be failing in AD. C. Increased intracellular Cu (and Zn, not shown here) leads to inhibited tau phosphorylation and, through JNK, increased matrix metalloproteinase 2&3 (MMP2/3) activities (White et al., 2006). D. Zn and Cu, liberated from aggregated Aß, increase the activities of interstitial metalloproteinases (Strozyk et al., 2007), such as neprilysin (NEP), insulin degrading enzyme (IDE), and tissue plasminogen activator (TPA). The increased amount of metalloproteinases accelerates the degradation of $Aß_{ISF}$.

[Figure S7]). Therefore, if tau reduction contributes to the cognitive improvements we observed, the mechanism may involve preventing tau (either phosphorylated or nonphosphorylated) from precipitating. The differential effect of CQ and PBT2 may be due to the increased brain penetration of PBT2, or its increased ionophoric activity and ability to impact upon tau phosphorylation, and warrants further investigation.

It is notable that despite the strong ionophoric properties of PBT2 in cell culture, treatment of Tg mice in these studies did not alter brain or peripheral metal levels. This is probably because PBT2 (and CQ) target Aß-bound metals that accumulate in a very small relative volume (e.g., 6% in 12-month-old APP/PS1 mice) (Stoltenberg et al., 2007) of the brain mass. Therefore, a shift of metals from extracellular Aß aggregates into the parenchymal cells may not be reflected as a change in total tissue metal levels, especially as homeostatic redistribution of the liberated metals takes effect. This is a reassuring element of the safety profile of the drug, since a perturbance of essential metal biochemistry is an undesirable effect of stronger chelators.

Similarly to our findings, the lipophilic bivalent metal ion modulator, DP-109, induced marked reductions in brain Aß after 3 months of treatment in the Tg2576 Tg line (Lee et al., 2004). However, it is not known whether this compound has ionophoric properties or if it improves cognitive performance. Genetic and pharmacological Cu augmentation in two APP Tg models, which reversed Cu deficiency induced by expression of the APP transgene, resulted in a significant decrease in amyloid burden (Bayer et al., 2003; Phinney et al., 2003). These reports support the hypothesis that perturbed metal homeostasis underlies disease progression in AD. However, oral Cu supplementation (8 mg/day) for 12 months was not able to prevent cognitive deterioration in a double-blind placebo-controlled trial in AD patients (http://www.alzheimer-bayer.de/alzh._st1.html, accessed May 18, 2008). We believe that compounds like CQ and PBT2 redistribute metals from extracellular collections and restore intracellular metal reserves where depleted, correcting not only the accumulation of extracellular amyloid but also correcting multiple biochemical deficits that are caused by dyshomeostasis of essential Cu and Zn (reviewed in Adlard and Bush [2006]).

Existing FDA-approved drugs for the treatment of AD are all based upon known neurotransmitter defects and are not considered to be disease-modifying. There are, however, several compounds in preclinical and clinical development that aim to lower Aß by a variety of mechanisms (Masters and Beyreuther, 2006). The studies reported here demonstrate rapid and pronounced benefits of ionophores on both AD-like neuropathology and cognitive dysfunction in Tg models of AD. While further investigations are required to test the model of mechanism of action, our findings support targeting synaptic metals, the factors that lead to Aß oligomerization and toxicity, as possibly a more potent disease intervention than systemically targeting the Aß molecule or its generation. Results of a recent phase IIa clinical trial in AD patients revealed a significant lowering of cerebral spinal fluid (CSF) Aß1-42 and an improvement in two subtests of the Neuropsychological Test Battery (NTB) within 3 months of oral treatment with PBT2. This further supports the predictive utility of our drug discovery approach. PBT2 is progressing as a potential disease-modifying treatment for AD.

EXPERIMENTAL PROCEDURES

Mice

Experimental groups of APP/PS1 [B6C3-Tg(APPswe,PSEN1 dE9)85Dbo/J] and WT littermates were utilized in this study. Animals were treated with CQ (30 mg/kg/d), PBT2 (10–30 mg/kg/d), or vehicle solution (SSV: 0.9% NaCl, 0.5% Na-carboxymethylcellulose, 0.5% benzyl alcohol, and 0.4% Tween-80) at 8 months of age for a total period of either 11 or 35 days (see Table S1 for outline), during which they were assessed in the MWM. The Tg2576 mouse line (encoding human APP695 with Lys670-Asn and Met671-Leu mutations) (Hsiao et al., 1996) was also used, and animals were studied at ~13.5 months of age. The Tg2576 mice were treated with either PBT2 (30 mg/kg/d) or SSV for a total period of 11 days, during which they were subject to assessment in the MWM. All drugs were administered daily by oral gavage, and group sizes were from between 6 to 18 animals per treatment. For

microdialysis experiments, cohorts of aged male and female APP/PS1 (~22 months old) and Tg2576 (~18 months) mice, in addition to young APP/PS1 (3.5 months) and young normal male C57Bl6/C3H (12 weeks) mice were used. Group sizes were between two and nine animals per treatment.

All experiments were conducted according to the Australian Code of Practice for the Use of Laboratory Animals and were approved by the Institutional Animal Experimentation Ethics Committee.

Ionophore Studies

M17 human neuroblastoma cells were plated overnight to 70% confluency (1 million cells) at the time of the experiment. All experiments were conducted in duplicate. Cells were incubated in 1 ml of Opti-MEM (Invitrogen) with added 10% FBS, Sodium Pyrvate, NEAA, and PenStrep (with or without varying concentrations of compound and 10 μM of Cu^{2+}, Zn^{2+}, or Fe^{3+} as chloride) for 5 hr at 37°C. At the end of the incubation, the media was removed and replaced with 1 ml PBS to dislodge the cells, which were then put into Eppendorf tubes and pelleted. The cell pellets were then used for inductively coupled plasma mass spectrometry (ICPMS) analysis of metal content as previously described (Maynard et al., 2006).

Dissolution of Zn-Induced Aß Precipitates

Aß1-42 (10 μM) was incubated with $ZnCl_2$ (20 μM) and thioflavin T (ThT) in a molar ratio of (1:2:2) for 24 hr at 37°C on a rotating wheel in PBS (pH 6.6). Following incubation, the mixture (containing peptide aggregates) was incubated with test compound for a further 2 hr at 37°C with rotation. CQ and PBT2 were dissolved in DMSO to a stock concentration of 5 mM. Dilutions were made in DMSO as appropriate to 100 times the desired final concentration, then added to the reaction buffer to a final DMSO concentration of 1% v/v PBS. Untreated aggregates and DMSO controls were included with each experiment. After 2 hr incubation, samples were measured for ThT fluorescence using an LS55 (Perkin Elmer) fluorimeter. Data were generated using FL Winlab software (Perkin Elmer). Each measurement was performed in triplicate.

Aß-Plaque-Associated Zn Removal

This technique was developed as a variation of previously published protocols (Lee et al., 2002), here used to directly assess the effects of PBT2 on amyloid-bound Zn. A female APP/PS1 animal and age-matched non-Tg littermate (~16 months old) were euthanized and the brains were immediately removed and sectioned on a vibratome (WPI Vibroslice) at 200 μm thickness. Multiple sections through the region occupied by the hippocampus were rinsed in PBS and then mounted on glass slides. Sections were then covered with the selective Zn stain Zinpyr-4 (NeuroBioTex, Inc, USA; 10 μM in PBS) for 3 min. Sections were then rinsed in PBS and viewed under the microscope. This stain labeled a significant number of plaques in each section from the Tg animal only, which were subsequently shown to be thioflavin-positive (1% solution in distilled water), confirming the specificity of the stain for Aß plaques. Individual plaques were imaged prior to application of PBT2 (n = 17 plaques), SSV control solution (n = 7), or 3-AQ (n = 12), and then the same plaques were reimaged 5 minutes later. The signal intensity of given plaques was assessed with the aid of a counting grid in ImageJ (version 1.38X), and the change in signal between baseline and 5 minutes postdrug was then quantitated and expressed as a percentage reduction in plaque fluorescence.

Inhibition of Catalytic H_2O_2 Generation by Aß:Cu Complexes

This technique was adapted from established protocols (Opazo et al., 2002) and is a dicholorofuoroscein (DCF)-based fluorometric assay that evaluates the ability of a test compound to inhibit H_2O_2 generated by Aβ complexed with Cu. DCF (5 mM in DMSO) (Molecular probes, Eugene, OR) is deacetylated in the presence of 0.25 M NaOH for 30 min and neutralized at pH 7.4 to a final concentration of 1 mM. Reactions are carried out in PBS (pH 7.4) in a 96-well plate. They contain Aß1-42 (50 nM to 1 μM), Cu-glycine chelate (Cu-Gly, prepared by adding $CuCl_2$ to glycine in the ratio of 1:6, then added to the Aß in the proportion 2Cu-Gly:1Aβ), a reducing agent (dopamine, 5 μM), deacetylated DCF 100 μM, and horseradish peroxidase (1 μM, pH 7.4). The reaction mixture is incubated at 37°C for 60 min. Positive controls are Cu alone and Aβ:Cu. The drugs alone or with Cu (2:1) do not generate or degrade H_2O_2. Fluorescence is recorded using a Perkin Elmer LS-55 fluorometer with excitation and emission filters at 485 nM and 530 nM, respectively. H_2O_2 concentration is established by comparing fluorescence with the H_2O_2 (1–2.5 μM) standards in PBS. Inhibition of H_2O_2 production is assayed by including selected concentrations of test compound in the wells. The criteria for acceptance of data are as follows: (1) test substance must not exhibit autofluorescence, (2) test substance must not generate intrinsic H_2O_2, and (3) test substance must not interfere with the detection system in any other way, e.g. inhibit peroxidase activity. PBT2 and CQ were both tested on three separate occasions and the Cu:drug complex was also shown to not react with H_2O_2.

Prevention of Cu-Induced Dityrosine Crosslinked Aß

10 μM Aß1-40 was incubated with 25 μM $CuCl_2$ and 50 μM ascorbic acid (molar ratio 1:2.5:5) for 1 hr at 37°C on a rotating wheel in PBS pH 7.4 (700 μl total volume). The reaction was stopped by the addition of 200 μM EDTA. The 1 hr reaction mixture without Cu constituted the negative control. In parallel reactions, CQ and PBT2 were incubated with the reaction components at various concentrations. The amount of dityrosine crosslinked Aß (Barnham et al., 2004) was assayed by densitometric quantification of ≈9 kDa bands of western blots probed with 1C3, a dityrosine-specific monoclonal antibody (a kind gift of Dr. Yoji Kato, Himeji Institute of Technology, Himeji, Japan).

Reversal of Aß-Induced Inhibition of LTP in Hippocampal Slices

C57Bl/6 mice (mixed gender, 14–40 days old) were decapitated under anesthesia by halothane inhalation and brains were rapidly removed and chilled in ice-cold artificial CSF (aCSF). Field potential recordings are made in 350 μm slices of the hippocampus by stimulating and recording in the stratum radiatum of the CA1 region of the hippocampus. Baseline stimulation intensity was that required to produce 20%–30% of the maximum slope of the field excitatory postsynaptic potential (fEPSP) and was calibrated at the beginning of each experiment. Tetanic stimulation was a 1 s 100 Hz pulse delivered in substitution for the test stimulus at the test intensity. Data were analyzed offline using pClamp 8.2. The slope of each fEPSP was determined by linear regression of the data points following the presynaptic fiber volley. LTP was quantified by averaging the data points from 55 to 60 min posttetanus for each slice. Aß1-42 (2 μM in aCSF) together with PBT2 or CQ were added to the slices for 30 min prior to commencing electrophysiological recordings.

Brain Uptake Studies

Mice were administered CQ or PBT2 (30 mg/kg) by oral gavage in 0.5% w/v hydroxypropylmethylcellulose, 0.5% v/v benzyl alcohol, and 0.4% v/v Tween 80. Mice were anesthetized with an intraperitoneal injection of ketamine and xylazine (133 mg/kg and 10 mg/kg, respectively) 4 min prior to blood and brain harvesting. At 120 min postdose (n = 4 or 5 mice at each time point), 500 μl of blood was withdrawn by cardiac puncture and transferred into tubes containing 10 μl of heparin (1000 l U/ml). Blood samples were immediately centrifuged to obtain duplicate 50 μl plasma samples. Immediately following blood collection, mice were sacrificed by cervical dislocation and the whole brain was removed. Brain and plasma samples were stored at −20°C for 12 days prior to analysis. On the day of analysis, the whole brain was homogenized in three parts of water, and an aliquot of the brain homogenate and plasma was analyzed for compound concentration by LCMS (Quattro Ultima Pt), with analytical lower limit of quantitation (LLQ) values for PBT2 at 1 ng/ml (plasma) and 10 ng/g (brain), and for CQ at 5 ng/ml (plasma) and 10 ng/g (brain). Standards were prepared by spiking blank brain homogenate or plasma. Both samples and standards were processed by adding acetonitrile to the tissue homogenate or plasma, and centrifuging and injecting an aliquot of the supernatant onto the LCMS. The concentration of each compound in the brain homogenate was corrected for the amount of compound remaining in the brain vasculature, the volume of which was previously determined by administration of the non-absorbable marker ^{14}C-inulin. Complete recovery (>90%) of PBT2 or CQ from the brain samples using the described extraction procedure was confirmed by spiking brain homogenate with either compound to a nominal concentration of 500 μg/l, and comparing the supernatant concentration following protein precipitation with that measured when the compounds were spiked into the same matrix following precipitation.

The plasma protein binding for CQ and PBT2 was assessed in mouse plasma. Aliquots of plasma (990 μl) were spiked with 10 μl of compound (in

DMSO) at a nominal concentration of 2 mg/l (final DMSO concentration 0.5%). Plasma samples were incubated at 37°C for 60 min before separating plasma proteins from plasma water by ultracentrifugation at 42,000 rpm (Beckman Rotor type 42.2 Ti) for 4 hr. To determine the fraction of each compound bound to plasma proteins, the concentrations in the supernatant (i.e., plasma water) and in uncentrifuged plasma control samples were determined by LCMS.

Behavioral Tests

We utilized the MWM to assess the effects of drug on spatial learning and memory, as previously described (Adlard et al., 2005). Briefly, after nonspatial pretraining, animals were subject to 6 days of place discrimination training, with four trials per day, followed by a probe trial 24 hr later to assess retention of the task. Data was analyzed using the Ethovision automated tracking system. There was no significant difference in the swim speed between the different groups of animals across the study.

IVM

Mice (APP/PS1 or Tg2576 animals) were sedated (atropine 0.5 mg/kg + xylazine 10 mg/kg) and then anesthetized (3%–4% isofluorane carried by oxygen). Following incisions in the scalp, a 0.75 mm bore hole was made above the right hippocampus. A sterile 27G guide cannula was then stereotaxically implanted into the hippocampus and held in place using anchoring bone screws and dental cement. The wound was then sutured around the implant and the animal was allowed to recover. The microdialysis probes (4 mm long, 38 kDa molecular weight cut off [MWCO] membrane; BASi and CMA), perfused with aCSF (pH 7.35), were inserted under light anesthesia. The animals were fitted with a mouse collar and then placed into individual housing chambers (Raturn Cage System, BioAnalytical Systems) where they remained for the duration of the experiment. Samples of interstitial fluid were automatically collected every hour via infusion lines into refrigerated sample collectors at a flow rate of 0.5 μl/min. Dialysates were then collected in 4 hr bins and subsequently lyophilized. The starting material and subsequent dialysis samples were western blotted with WO2 antibody and quantitated against Aß1-42 standards. The identity of the $Aß_{ISF}$ immunoreactivity was confirmed by probing with other Aß antibodies, 4G8 and 6E10. The efficiency of recovery of Aß utilizing this method was assessed by passing aCSF through a 38 kDa MWCO microdialysis probe immersed in a soluble brain fraction (where the brain was sonicated in PBS and centrifuged at 100,000 × g for 30 min, and the supernatant was removed) of a 12-month-old female APP/PS1 mouse. The percentage recovery of WO2 reactive Aß species in the dialysate was calculated by ([D/S]×100), where D is the concentration of Aß in the dialysate at a given flow rate and S is the concentration of Aß in the starting material. The efficiency of our system was 8.2% ± 1.8% (SEM, n = 6), indicating that either much of the Aß in the brain soluble fraction exists as homo- or hetero-oligomers of >38 kDa in size, or that much of the Aß adheres to surfaces or is degraded in passage through the tubing.

Baseline samples were collected for a period of 8 hr prior to oral gavage of drug. Drug (PBT2 or CQ) was administered and then samples were collected for 20 hr.

Tissue Preparation

At the completion of all animal studies (those incorporating behavioral assessment), mice were anesthetized (Lethabarb: pentobarbitone sodium, Virbac) and transcardially perfused with ice-cold PBS. The brain was then removed, split into its hemispheres, and frozen on dry ice. The tissues were later thawed, homogenized (by sonication) in PBS (containing protease inhibitor cocktail, Sigma), and centrifuged at 100,000 × g (4°C, 1 hr) to collect pellet and supernatant (soluble) fractions. These were subsequently used for all biochemical assessments.

SELDI-TOF Mass Spectrometry

PS10 ProteinChip arrays were used for all experiments. Antibody (4G8 in PBS [0.25 g/l]) and negative control (bovine IgG at the same concentration and volume) were incubated on the chips in a humidity chamber at 4°C overnight. The antibodies were then removed and blocking buffer (5 μl, 0.5 M ethanolamine in PBS) incubated on the chip for 60 min. This was then removed and each spot was washed with 20 μl of 0.5% Triton X-100/PBS (wash buffer) for 5 min and then 20 μl of PBS for 5 min. Samples were prepared by adding 1 μl of Triton X-100 and 12.5 μl of 8 M urea to 100 μl of each sample and then mixing. Fifteen microliters was then added to each spot and the array was incubated at room temperature (RT) for three hours. The samples were removed and each spot was rinsed with wash buffer (2 × 50 μl, 10 s), followed by a wash with PBS (2 × 50 μl, 10 s), and finally, the arrays were washed with 1 mM HEPES (2 × 50 μl, 10 s) and air-dried. All the incubations and washes were performed on a shaking table. Sinapinic acid (50% [v/v] acetonitrile/0.5% TFA) was applied to each spot twice, with the chip air-dried between each application. Analysis was conducted using a PBSIIc, SELDI-TOF mass spectrometry, and peaks were analyzed using Ciphergen ProteinChip software 3.1.

Western Blot

Western blot was utilized to assess total tau (anti-human total tau, DAKO), phosphorylated tau (tau phosphorylated at Ser396, Biosource), synaptophysin (Chemicon), dityrosine (mAb 1C3 [a gift from Dr. Yoji Kato, Himeji Institute of Technology, Himeji, Japan]), soluble oligomeric Aß using antibody A11 (Kayed et al., 2003) (a gift from Dr. Charles Glabe, University of California at Irvine, USA), APP (22C11, Chemicon), and total Aß (WO2, 6E10, 4G8). Samples were run on NuPage NOVEX Invitrogen bis-tris 4%–12% 26-well gels at 160 V for 1 hr. Membranes were heated in PBS (5 min, microwave), blocked in TBST (0.01% Tween, 3% BSA), and incubated with antibody overnight (4°C). Blots were rinsed in TBST and then incubated in secondary antibody (1 hr, RT), followed by further rinsing, development with ECL reagent, and imaging using a Las-3000 image capture system (Fujifilm).

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/59/1/43/DC1/.

ACKNOWLEDGMENTS

This study was funded by Prana Biotechnology Ltd, as well as funds from the Australian Research Council (to A.I.B.), the National Health and Medical Research Council (P.A.A., R.C., and K.J.B. supported by fellowships) and the Alzheimer's Association (to P.A.A., D.I.F., and A.I.B.). We thank Francois Monty (MHRI), Edmund Kostewicz (CDCO), and Julia Morizzi (CDCO) for technical assistance and Dr. Axel Unterbeck for helpful comments. We are grateful to Penny Huggins, Jack Parsons, Vijaya Kenche, Darryl Carrington, and Stephanie Toh for their involvement in the development of PBT2.

Drs. Adlard, Cherny, Finkelstein, Cappai, Ritchie, Tanzi, Masters, Barnham, and Bush are consultants to Prana Biotechnology Ltd; Dr. Gautier is a paid employee of Prana Biotechnology Ltd.

Received: December 5, 2007
Revised: May 13, 2008
Accepted: June 25, 2008
Published: July 9, 2008

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